(ii) Conditional Toxic Palliatives

Another approach for inhibiting a pathogenic agent is to express a palliative which is toxic for the cell expressing the pathogenic condition. In this case, expression of the palliative from the proviral vector should be limited by the presence of an entity associated with the pathogenic agent, such as an intracellular signal identifying the pathogenic state in order to avoid destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection by targeting recombinant retrovirus carrying the vector to cells having or being susceptible to the pathogenic condition.

In one embodiment of this method, a recombinant retrovirus (preferably, but not necessarily, a recombinant MLV retrovirus) carries a vector construct containing a cytotoxic gene (such as ricin) expressed from an event-specific promoter, such as a cell cycle-dependent promoter (e.g., human cellular thymidine kinase or transferrin receptor promoters), which will be transcriptionally active only in rapidly proliferating cells, such as tumors. In this manner, rapidly replicating cells, which contain factors capable of activating transcription from these promoters, are preferentially destroyed by the cytotoxic agent produced by the proviral construct.

In a second embodiment, the gene producing the cytotoxic agent is under control of a tissue-specific promoter, where the tissue specificity corresponds to the origin of the tumor. Since the viral vector preferentially integrates into the genome of replicating cells (for example, normal liver cells are not replicating, while those of a hepatocarcinoma are), these two levels of specificity (viral integration/replication and tissue-specific transcriptional regulation) lead to preferential killing of tumor cells. Additionally, event-specific and tissue-specific promoter elements may be artificially

combined such that the cytotoxic gene product is expressed only in cell types satisfying both criteria (e.g., in the example above, combined promoter elements are functional only in rapidly dividing liver cells). Transcriptional control elements may also be amplified to increase the stringency of cell-type specificity.

These transcriptional promoter/enhancer elements need not necessarily be present as an internal promoter (lying between the viral LTRs) but may be added to or replace the transcriptional control elements in the viral LTRs which are themselves transcriptional promoters, such that condition-specific transcriptional expression will occur directly from the modified viral LTR. In this case, either the condition for maximal expression will need to be mimicked in retroviral packaging cell lines (e.g., by altering growth conditions, supplying necessary transregulators of expression or using the appropriate cell line as a parent for a packaging line), or the LTR modification is limited to the 3' LTR U3 region, to obtain maximal recombinant viral titres. In the latter case, after one round of infection/integration, the 3' LTR U3 is now also the 5' LTR U3, giving the desired tissue-specific expression.

In a third embodiment, the proviral vector construct is similarly activated but expresses a protein which is not itself cytotoxic, and which processes within the target cells a compound or a drug with little or no cytotoxicity into one which is cytotoxic (a "conditionally lethal" gene product). Specifically, the proviral vector construct carries the herpes simplex virus thymidine kinase ("HSVTK") gene downstream and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Expression of the tat gene product in human cells infected with HIV and carrying the proviral vector construct causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to

a drug such as acyclovir or its analogues (FIAU, FIAC, DHPG). These drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms (see, for example, Schaeffer et al., Nature 272:583, 1978). Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., PNAS, Natl. Acad. Sci. USA 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs. In addition, an extra level of specificity is achieved by including in the vector the HIV rev protein, responsive CRS/CAR sequences. In the presence of the CRS sequence gene expression is suppressed, except in the presence of the CAR sequences and the rev protein. Example 5 provides an illustration of this technique.

EXAMPLE 5

Vector to Conditionally Potentiate the Toxic Action of ACV or Its Analogues

Construction of Vectors

A. Construction of pKTVIHAX (see Figure 7).

1. The 9.2 kb Asu II/Xho I fragment was isolated from vector pN2 DNA.

2. The 0.6 kb Xho I/Bam HI promoter fragment was isolated from plasmid pSKHL.

3. The 0.3 kb Bgl II/Acc I and 1.5 kb Acc I/Acc I fragment were purified from pUCTK.

4. The fragments from 1, 2, and 3 were ligated, transformed into bacteria, and appropriate Amp^R clones of the given structure identified by restriction enzyme analysis.

B. Construction of pKTVIH-5 and pKTVIH5 Neo retroviral vectors (see Figure 8).

1. The 4.5 kb 5' LTR and vector fragment was isolated as an Xho I/Bam HI fragment from vector p31N25delta(+).

2. The 1.0 kb 3' LTR was isolated as an Apa I/Bam HI fragment from pN2R3(+) fragment.

3. The 0.6 kb HIV promoter element was isolated from pSKHL as an Apa I/Eco R1 fragment.

4. The HSVTK coding sequence and transcriptional termination sequences were isolated as 1.8 kb Eco R1/Sal I fragment from pUCTK.

5. The fragments from 1-4 were combined, ligated, transformed into bacteria, and clones of the given structure were identified by restriction enzyme analysis (pKTVIH-5).

6. Plasmid pKTVIH5 Neo was constructed by linearizing pKTVIH5 with Cla I; mixing with a 1.8 kb Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, and Tn5 Neo^{R} marker, ligating, transforming bacteria and selecting for kanamycin resistance. Clones with the insert in the indicated orientation were identified by restriction analysis.

C. Construction of MHMTK Neo retroviral vector (see Figure 9).

1. Construction of intermediate plasmid MHM-1 LTR.

a) Plasmid pN2CR5 was linearized by partial digestion with Fok I, the 5' overhang filled in with deoxynucleotide triphosphates using Klenow DNA polymerase, and Hind III linkers inserted. After transformation into bacteria, a clone with a Hind III linker inserted in the MLV LTR Fok I site was identified by restriction enzyme analysis (pN2CR5FH).

b) Plasmid pN2CR5FH was linearized with Nhe I, the 5' overhang filled in with Klenow

polymerase digested with Hind III, and the 4.3 kb fragment with promoterless MLV sequences isolated.

c) 0.5 kb Eco RV/Hind III HIV promoter sequences were isolated from pSKNL.

d) b and c were mixed, ligated, used to transform bacteria, and the structure of MHM-1 was confirmed by restriction enzyme analysis.

2. The 0.7 kb Eco RV/Bal I fragment isolated from MHM-1 was subcloned into the Eco RV site of plasmid IBI30B (a modified IBI30 plasmid containing additional Bgl II, Bst II, Neo I and Nde I sites in the polylinker). After transformation into bacteria, clones with the appropriate orientation were identified by restriction enzyme analysis (pMHMB).

3. Plasmid pMHMB was digested with Apa I and Xho I and gel purified.

4. MHM-1 was digested with Apa I/Bam HI and the 1.8 kb MHMLTR/leader sequence gel purified.

5. The 2.8 kb Bgl II/Sal I fragment containing the HSVTK coding region upstream of the SV40 early promoter driving Neo^r taken from pTK-3 (see Figure 3).

6. 3-5 were mixed, ligated, used to transform bacteria, and appropriate clones were identified by restriction enzyme analysis.

This vector and similar vectors which contain inducible elements in their LTR's result in an added safety feature. Briefly, since the LTR is inactive in the absence of HIV, insertional downstream activation of undesirable host genes (such as proto-oncogenes) does not occur. However, tat expression in the packaging cell line allows facile manipulation of the virion in tissue culture.

D. Construction of BRKTVIH retroviral vector (see Figure 10)

1. The 9.2 kb Asu II/Xho I fragment was isolated from vector pN2 DNA.

2. The 0.6 kb Xho I/Eco R1 HIV promoter fragment was isolated from plasmid pSKHL.

3. The HIV rev responsive HSVTK (RRTK) was constructed in the following manner:

a) The HSVTK gene was subcloned as a 1.8 kb HinC II/Pvu II fragment into the Eco RV site of vector SK$^+$ (pSTK(-)).

b) The 1.8 kb Kpn I/Hind III fragment which contains the CRS/CAR elements from HIV env was repaired and blunt-end ligated into the Sma I site of vector I30B (pCRS/CAR(+/-)). I30B is a modified IBI30 plasmid containing the same additional restriction sites as for pUC31 with an Nde I site instead of the IBI30 Xho I site.

c) The 3.6 kb BssH II/Eco R1 fragment containing vector and HSVTK polyadenylation signals was isolated from pSTK(-),

d) The 1.8 kb Bam HI/BssH II CRS/CAR fragment was isolated from pCRS/CAR(-).

e) The 1.2 Eco R1/Bam HI coding sequence fragment was isolated from pTKdeltaA.

f) C, D and E were ligated and appropriate recombinants screened by restriction enzyme analysis.

4. Rev-responsive HSVTK was isolated as a 3.6 kb Eco R1/Cla I fragment.

5. 1, 2 and 4 were ligated and appropriate recombinants identified by restriction enzyme analysis.

E. Construction of tat and anti-tat expression vectors (see Figure 11).

These vectors are used as pseudo-HIV to test-activate tat-dependent HSVTK vectors.

1. The His$^-$ expression vector pBamHis was linearized with Bam HI and treated with calf intestinal phosphatase.

2. The Sac I site of pCV-1 was mutagenized to a Bam HI site and the 380 bp Bam HI coding sequence of HIV tat was isolated.

3. The fragments purified in steps 1 and 2 were mixed, ligated, used to transform bacteria, and clones with tat in both orientations (expressing tat or the "anti-sense" tat) were identified by restriction enzyme analysis.

These constructs were used to generate infectious recombinant vector particles in conjunction with a packaging cell line such as PA317, as described above. These vectors are genetically stable and result in predictable proviral structure as judged by Southern blot analysis of restriction-enzyme-digested genomic DNA from individual clones of infected cells (39/40 clones tested had proviruses of the expected size).

The biological properties of these retroviral vectors are described hereinafter. The HIV tat gene ("tathis" vector -- see Figure 11) was transfected into mouse PA317 cells. Five individual histidinol-resistant subclones were obtained (TH 1-5) which express HIV tat. These cells are thus an experimental model for HIV infection. The vectors KTVIHAX, KTVIH5NEO, and MHMTKNEO, were subsequently introduced by infection into these tat-expressing cell lines as well as their parent cell line lacking tat. Cell viability was then determined in various concentrations of the HSVTK-specific cytotoxic drug, acyclovir (ACV). The data are reported here as LD50 (the drug concentration at which 50% toxicity is observed). The parental cell line containing the vector but lacking tat (non-HIV-infected model) showed no detectable toxicity by ACV at the concentrations tested (see Figure 12). These cells thus require 100 uM ACV or greater for cytotoxicity. This is true also for these cells lacking the vectors. Thus the vectors alone, ACV alone, or even the vector +ACV (solid boxes) is not cytotoxic. However, cell lines which express HIV tat (the

experimental representation of an HIV infection) are effectively killed by ACV. This is true to varying degrees for all three vectors tested. These data indicate that HIV-infected cells will be killed in the presence of ACV and "potentiator" vectors.

In an analogous experiment, vectors KTVIHAX and KTVIHS Neo were introduced by infection into human T-cell and monocyte cell lines Sup T1, HL60 and U937 cells. Subsequently, these cells were infected with tat his or rtat vectors, selected in histidinol, and cell viability determined at various concentrations of the ACV analog, FIAU. The LD_{50} reported in Table 1 (below) indicate that a vector dependent increase in FIAU toxicity occurs in the absence of HIV tat but is increased an additional ten- to twentyfold when tat is present. This indicates that although there is a baseline HSVTK expression in all but HL60 cells, expression is even greater in the presence of HIV tat.

TABLE 1

HIV tat inducibility of FIAU cytotoxicity in human monocyte and T-cell lines infected with conditionally lethal recombinant retroviral vectors

Cell Type	Vectors	tat	LD50FIAU(μM)
HL60 ("monocyte")	--	-	50
	--	+	50
	KTVIHAX	-	50
	KTVIHAX	+	<0.2
	KTVIH5Neo	-	50
	KTVIH5Neo	+	<0.2
U937 ("monocyte")	--	-	10
	KTVIHAX	-	0.5
	KTVIHAX	+	0.05
	KTVIH5Neo	-	0.5
	KTVIH5Neo	+	0.05
Sup T1 ("T-cell")	--	-	10
	--	+	5
	KTVIHAX	-	0.5
	KTVIHAX	+	0.05
	KTVIH5Neo	-	0.5
	KTVIH5Neo	+	0.05
H9 ("T-cell")	--	-	10
	KTVIHAX	-	2
	KTVIHAX	+	0.2
	KTVIH5Neo	-	1
	KTVIH5Neo	+	0.08

Similarly, HIV infection of human T-cell line H9 +/- FIAU show a fivefold preferential inhibition (through cell killing) of HIV infection. Cultures were first treated with vector, then challenged with HIV for 4 days. Viral supernatants were then titred using the HIV assay, as described in Section IV.

In the case of HIV-infected cells, expression of the conditionally lethal HSVTK gene may be made even more HIV-specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., Proc. Natl. Acad. Sci. USA 85:2071, 1988). Such a tat- and rev-responsive vector (RRKTVIH) has been constructed (see Figure 10) and amphotrophic virus has been generated.

More generally, cis elements present in mRNAs have been shown in some cases to regulate mRNA stability or translatability. Sequences of this type (i.e., post-transcriptional regulation of gene expression) may be used for event- or tissue-specific regulation of vector gene expression. In addition, multimerization of these sequences (i.e., rev-responsive "CRS/CAR" or tat-responsive "TAR" elements for HIV) could result in even greater specificity. It should be noted that this kind of conditional activation of an inactive precursor into an active product in cells may also be achieved using other viral vectors with a shorter term effect, e.g., adenovirus vectors. Such vectors are capable of efficiently entering cells and expressing proteins encoded by the vector over a period of time from a couple of days to a month or so. This period of time should be sufficient to allow killing of cells which are infected by both HIV and the recombinant virus, leading to HIV dependent activation of expression of a gene carried by the recombinant virus. This gene expression would then allow conversion of an inactive precursor into an active (e.g., lethal) product.

Production, concentration and storage of vector preparations is as previously described. Administration is by direct in vivo administration as before or by ex corpora treatment of PBL and/or bone marrow. Doses will be at approximately the same levels as for Example 4. Targeting of viral vector infection will not be through the CD4 receptor, but may be accomplished through producing vector particles which will infect cells using the HIV env protein (gp120) as a receptor. Such HIV-tropic viruses may be produced from an MLV-based packaging cell line constructed from cells which have naturally high levels of CD4 protein in their cell membrane (for example, Sup T1 cells) or from any cell type "engineered" to express the protein. The resultant virions, which form by budding from the cell membrane itself, contain the CD4 protein in their membrane. Since membranes containing CD4

are known to fuse with membranes carrying HIV env, these virions should fuse with cells containing HIV env and result in the specific infection of HIV-infected cells which have gp120 on their surface. Such a packaging cell line may require the presence of an MLV env protein to allow proper virion assembly and budding to result in infectious virions. If so, an MLV env which does not infect human cells (such as ecotropic env) would be used such that viral entry will occur only through the CD4/HIV env interaction and not through the MLV env cell receptor, which would presumably not depend upon the presence of HIV-env for infection. Alternatively, the requirement for MLV env may be satisfied by a hybrid envelope where the amino-terminal binding domain has been replaced by the amino-terminal HIV-env binding domain of CD4. This inversion of the normal virus-receptor interaction can be used for all types of viruses whose corresponding cellular receptor has been identified.

In a similar manner to the preceding embodiment, the retroviral vector construct can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium, fungus, or protozoan. An example of this would be the E. coli guanine phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (see Besnard et al., Mol. Cell. Biol. 7:4139-4141, 1987). Conditionally lethal gene products of this type have potential application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., Brit. J. Cancer 53:377-384, 1986).

Mammalian viruses in general tend to have "immediate early" genes which are necessary for subsequent transcriptional activation from other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes from transcriptional promoter elements responsive to these viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human α and β interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VRE) could result in the destruction of cells infected with a variety of different viruses.

In a fourth embodiment, the recombinant retrovirus carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant retrovirus could carry a gene encoding a proprotain for ricin A chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

In a fifth embodiment, the retroviral construct may express a "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as HIV tat protein). This surface protein can be recognized by a cytotoxic agent, such as antibodies for the reporting protein or by cytotoxic T-cells. In a similar manner, such a system can be used as a detection system (see below) to simply

identify those cells having a particular gene which expresses an identifying protein, such as the HIV tat gene.

Similarly, in a sixth embodiment, a surface protein could be expressed which would itself be therapeutically beneficial. In the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

EXAMPLE 6

Construction of p4TVIHAX retroviral vector

(see Figure 12)

1. The 9.4 kb Asu II/Xho I fragment was isolated from pM2.

2. The 0.6 kb Xho I/Eco R1 HIV promotor fragment was isolated from pSKHL.

3. The 1.4 kb coding region for human CD4 was isolated as an Eco R1/Bst Y1 (Xho II) fragment from the expression vector, pMV7T4.

4. The (A)n signal of HSVTK was isolated as a 0.3 kb Apa I/Sma I fragment, 3' repaired with T4 polymerase and dNTP's and cloned into the Sma I site of pUC31. After transforming bacteria, clones were screened for orientation by restriction enzyme analysis (p31[A]n[+/-]). The 0.3 kb (A)n signal was then isolated as a 0.3 kb Bgl II/Acc I fragment.

5. 1-4 clones were mixed, ligated, used to transform bacteria and clones were identified by restriction enzyme analysis.

Recombinant amphotrophic retroviruses have been produced and introduced into human monocyte and T-cell lines lacking or containing the HIV tat expression vector, tathis. Syncytia assays with HIV env-expressing mouse fibroblasts show that monocyte cell lines HL60 and U937 themselves lack sufficient CD4 to fuse with these cells. However, HL60 and U937 cells containing vector 4TVIHAX can fuse with the reporter cells (HIV-env expressing cells) when HIV tat is present, but not in its absence. These data indicate that CD4 expression is inducible and biologically active (as judged by syncytia formation). Experiments with the vector in human T-cell line, H9, indicated exceptionally high toxicity due to HIV infection and a correspondingly low HIV titre (more than 200-fold lower than the HIV titre produced in H9 cells lacking the vector).

In a seventh embodiment, the retroviral vector codes for a ribozyme which will cleave and inactivate RNA molecules essential for viability of the vector infected cell. By making ribozyme production dependent on an intracellular signal corresponding to the pathogenic state, such as HIV tat, toxicity is specific to the pathogenic state.

IV. Immune Down-Regulation

As briefly described above, the present invention provides recombinant retroviruses which carry a

vector construct capable of suppressing one or more elements of the immune system in target cells infected with the retrovirus.

Specific down-regulation of inappropriate or unwanted immune responses, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products which suppress surface expression of transplantation (MHC) antigen. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells and prevents terminal glycosylation and translocation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with gp 19-encoding vector constructs which upon expression of the gp 19 inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythromiatis, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

An alternative method involves the use of anti-sense message, ribozyme, or other specific gene expression inhibitor specific for T-cell clones which are autoreactive in nature. These block the expression of the T-cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using the viral vector delivery system.

V. Expression of Markers

The above-described technique of expressing a palliative in a cell, in response to some identifying agent, can also be modified to enable detection of a particular gene in a cell which expresses an identifying protein (for example, a gene carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells carrying an identifying protein associated with the virus.

This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), and carrying a promoter, which responds to the presence of the identifying protein in indicator cells, by switching expression of the reporting product between expressing and nonexpressing states. For example, HIV, when it infects suitable indicator cells, makes tat and rev. The indicator cells can thus be provided with a genome (such as by infection with an appropriate recombinant retrovirus) which codes for a marker gene, such as the alkaline phosphatase gene, β-galactosidase gene or the luciferase gene, and a promoter, such as the HIV promoter, which controls expression of the marker gene. When the indicator cells are exposed to a clinical sample to be tested, and the sample contains HIV, the indicator cells become infected with HIV, resulting in tat and/or rev expression (an identifying protein) therein. The HIV expression controls in the indicator cells would then respond to tat and/or rev proteins by switching expression of genes encoding β-galactosidase, luciferase, or alkaline phosphatase (marker products) from normally "off" to "on." In the case of β-galactosidase or alkaline phosphatase, exposing the cells to substrate analogues results in a color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is

positive for HIV. For intracellular enzymes such as β-galactosidase, the viral titre can be measured directly by counting colored or fluorescent cells, or by making cell extracts and performing a suitable assay. For the membrane bond form of alkaline phosphatase, virus titre can also be measured by performing enzyme assays on the cell surface using a fluorescent substrate. For secreted enzymes, such as an engineered form of alkaline phosphatase, small samples of culture supernatant are assayed for activity, allowing continuous monitoring of a single culture over time. Thus, different forms of this marker system can be used for different purposes. These include counting active virus or sensitively and simply measuring viral spread in a culture and the inhibition of this spread by various drugs.

Further specificity can be incorporated into the preceding system by testing for the presence of the virus either with or without neutralizing antibodies to that virus. For example, in one portion of the clinical sample being tested, neutralizing antibodies to HIV may be present; whereas in another portion there would be no neutralizing antibodies. If the tests were negative in the system where there were antibodies and positive where there were no antibodies, this would assist in confirming the presence of HIV.

Within an analogous system for an in vitro assay, the presence of a particular gene, such as a viral gene, may be determined in a cell sample. In this case, the cells of the sample are infected with a suitable retroviral vector which carries the reporter gene linked to the expression controls of the virus of interest. The reporter gene, after entering the sample cells, will express its reporting product (such as β-galactosidase or luciferase) only if the host cell expresses the appropriate viral protains.

These assays are more rapid and sensitive, since the reporter gene can express a greater amount of

reporting product than identifying agent present, which results in an amplification effect. Example 7 describes a representative technique for detecting a gene which expresses an identifying protein.

EXAMPLE 7

HIV-Specific Marker System or Assay

A. Constructs

Reporter constructs under the control of the HIV expression system are shown in Figure 14 (a recombinant retroviral vector) and in Figure 15 (a simple plasmid used by transfection). The pieces of these preferred vector and plasmid reporters were derived as follows:

The retroviral backbone was derived from the construct pAFVXM (Kriegar et al., Cell 38:384, 1984), which had been linearized using Xho I and Cla I. SV_2neo was obtained from the plasmid pKoneo (Hanahan, unpubl.) by isolation of the 1.8 kb Cla I fragment.

The HIV LTR was isolated as a 0.7 kb Hind III fragment from the plasmid pC15CAT (Arya et al., Science 229:69, 1985). Beta-gal was obtained from the plasmid pSP65 β-gal (Cepko, pers. comm.) as a Hind III-Sma I fragment. A secreted form of human placental alkaline phosphatase was produced by introduction of a universal terminator sequence after amino-acid 489 of the cell surface form of alkaline phosphatase (as described by Berger et al., Gene 66:1, 1988). The secreted alkaline phosphatase gene was isolated as a 1.8 kb Hind III to Kpn I fragment. The CRS-CAR sequences from HIV env were obtained by isolating the 2.1 kb Kpn I to Bam HI fragment from HTLVIIIB/BH10R3 (Fisher et al., Science 233:655, 1986). This fragment was inserted into pUC21 linearized by Bam HI, and Kpn I pUC21 is pUC19 (Yanisch-Perron et al., Gene 33:103, 1985) with extra Xho I, Bgl II, Bssh II and Nco I sites between the Eco RI and Kpn I sites of pUC19. The Bam HI site of the resulting construct was

converted to a Nco I site to allow resection of the CRS-CAR sequences by Nco I digestion. The SV40 t intron was obtained from pSVOL (de Wet et al., Mol. Cell. Biol. 7:725, 1987) as a 0.8 kb Nco I to Bam HI fragment.

B. Indicator Cells and Retroviral Vectors

Human T-cell (H-9, CEM and Sup T1) and monocyte (U-937) cell lines were obtained from ATCC, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

The nonretroviral vectors were introduced into cell lines by electroporation using a Bio-Rad Gene Pulser. The cell lines were selected in G-418 (1 mg/ml) for 2-3 weeks to obtain stable $G\text{-}418_R$ cell lines, and then dilution cloned to obtain clonal cell lines.

The pAF vectors were transfected into the PA317 packaging cell line as a calcium phosphate precipitate (Wigler et al., Cell 16:777, 1979). The virus-producing PA317 cells were co-cultivated with human monocyte cell lines for 24 hours in the presence of polybrene, after which the suspension cells were removed and selected in G-418 and subcloned as above.

C. Assay

Stable cell lines were infected with HIV ($HTLV\ III_B$) and the cells (β-gal) or media (alkaline phosphatase) assayed on a daily basis for 6 days post-infection.

β-Galactosidase Assay

Infected cells could be assayed by either: (i) In situ histochemical staining as described by MacGregor et al. Somatic Cell and Mol. Genetics 13:253, 1987); or (ii) by using cell extracts in a solution enzymatic assay with ONPG as a substrate (Norton and Coffin, Mol. Cel. Biol. 5:281, 1985).

Soluble Alkaline Phosphatase Assay

Medium was removed from infected cells, microfuged for 10 seconds, and then heated to 68°C for 10 minutes to destroy endogenous phosphatases. The medium was then microfuged for 2 minutes and an aliquot (10-50 µl) removed for assay. 100 µl of buffer (1 M diethanolamine, pH 9.8; 0.5 Mm $MgCl_2$; 10 mM L-homoarginine) was added and then 20 µl of 120 mM p-nitrophenylphosphate (in buffers) was added. The A_{405} of the reaction mixture was monitored using an automatic plate reader.

Figures 16 and 17 depict typical results of a time course of infection of Sup T1 cells using the alkaline phosphatase assay in the presence of varying concentrations of antiviral drugs. The "+" and "-" on day 6 indicate the presence or absence of syncytia.

The present invention provides a number of other techniques (described below) which can be used with the retroviral vector systems employed above, so as to enhance their performance. Alternatively, these techniques may be used with other gene-delivery systems.

VI. Packaging Cell Selection

This aspect of the present invention is based, in part, upon the discovery of the major causes of low recombinant virus titres from packaging cells, and of techniques to correct those causes. Basically, at least five factors may be postulated as causes for low recombinant virus titres:

1. the limited availability of viral packaging proteins;

2. the limited availability of retroviral vector RNA genomes;

3. the limited availability of cell membrane for budding of the recombinant retroviruses;

4. the limited intrinsic packaging efficiency of the retroviral vector genome; and

5. the density of the receptor specific for the envelope of a given retrovirus.

As noted above, the limited availability of viral packaging proteins is the initial limiting factor in recombinant retrovirus production from packaging cells. When the level of packaging protein in the packaging cells is increased, titre increases to about 10^5 infectious units/milliliter, following which increasing packaging protein level has no further effect on titres. However, titres can be further augmented by also increasing the level of retroviral vector genome available for packaging. Thus, as described herein, it is advantageous to select producer cells that manufacture the maximum levels of packaging proteins and retroviral vector genomes. It has been discovered that the methods of identifying, and thus selecting, packaging cells and producer cells, described earlier under the section entitled "Background of the Invention," tend to lead to selection of many producer cells which produce low titres for the reasons described below.

The present invention takes advantage of the previously disadvantageous fact that the protein expression level of a gene downstream from the 5' LTR or other promoter, and spaced therefrom by an intervening gene, is substantially less than if the intervening gene were absent. In the present invention, the selectable gene is placed downstream from a gene of the packaging genome or the gene of interest carried by the vector construct, but is still transcribed under the control of the viral 5' LTR or other promoter without any splice donor or splice acceptor sites. This accomplishes two things. First, since the packaging genes or genes of interest are now upstream with no intervening gene between themselves and the promoter, their corresponding proteins (packaging protein or protein of interest) will be expressed at a higher level (five- to twentyfold) than the selectable protein. Second, the selectable protein will

be expressed on average at a lower level, with the distribution of level of expression shifting toward lower levels. In the case of the phleo^{r} protein, this shift in distribution is illustrated by the broken curve indicated in Figure 18. However, the selection level for resistance to phleomycin remains the same, so that only the top-end expressing cells survive. The levels of the packaging protein or of the protein of interest will still be proportional, only in this case, a higher level of selectable protein corresponds to a much higher level of packaging protein or protein of interest.

Preferably, the foregoing procedure is performed using a plasmid carrying one of the proviral gag/pol or env packaging genes, along with a first selectable gene. These cells are then screened for the cells producing the highest levels of protein by reaction with an antibody against env (or possibly gag/pol), a second fluorescent antibody, and then sorted on a fluorescence-activated cell sorter (FACS). Alternatively, other tests for protein level may be used. Subsequently, the procedure and screening are repeated using those selected cells, and the other of the gag/pol or env packaging genes. In this step, a second selectable gene (different from the first) would be required downstream from the packaging gene and the cells producing the largest amount of the second viral protein selected. The procedure and screening are then repeated using the surviving cells, with a plasmid carrying the proviral vector construct bearing the gene of interest and a third selectable gene, different from the first or second selectable gene. As a result of this procedure, cells producing high titres of the desired recombinant retrovirus will be selected, and these can be cultured as required to supply recombinant retrovirus. In addition, gag and pol can be independently introduced and selected.

Example 8 describes the construction of gag/pol and env plasmids designed to use these procedures.

EXAMPLE 8

Plasmids Designed to Make High Levels of Packaging Proteins (Figure 19)

1. The 2.7 kb Xba I fragment from pPAM (Miller et al., Mol. Cell. Biol. 5:431, 1985), which contains the amphotrophic env segment, was cloned in pUC18 at the Xba I site, then removed with Hind III and Sma I. This fragment was cloned into the vector pRSV neo (Gorman et al., Mol. Cell. Biol. 2:1044, 1982; Southern et al., J. Mol. Appl. Genet. 1:327, 1982) cut with Hind III and Pvu II, to give pRSV env. A 0.7 kb Bam HI to BstE II fragment from the plasmid pUT507 (Mulsant et al., Somat. Cell. Mol. Genet. 14:243, 1988) with the BstE II end filled in carries the phleo resistance coding sequence. The 4.2 kb Bam HI to Xho I fragment, the contiguous 1.6 kb Xho I to Xba I (Xba I filled in) from RSVenv, and the phleo fragment were ligated to give pRSVenv-phleo.

2. A fragment from the Pst I site at nucleotide 563 of MLV (RNA Tumor Viruses, Vol. II, Cold Spring Harbor, 1985) to the Sca I site at 5870 was derived from pMLV-K (Miller et al., 1985, op. cit.) and cloned in the Pst I to Bam HI (Bam HI filled-in) fragment from p4aA8 (Jolly et al., Proc. Natl. Acad. Sci. USA 80:477, 1983) that has the SV40 promoter, the pBR322 ampicillin resistance and origin of replication and the SV40 poly A site. This gives pSVgp. pSVgpDHFR was made using the following fragments: the 3.6 kb Hind III to Sal I fragment from pSVgp containing the SV40 promoter plus MLV gag and some pol sequences; the 2.1 kb Sal I to Sca I fragment from pMLV-K with the rest of the pol gene, the 3.2 kb Xba I (Xba I filled-in) to Pst I fragment from pF400 with the DHFR gene plus poly A site, pBR322 origin and half the ampicillin resistance gene; the 0.7 kb Pst I to Hind III fragment from pBR322 with the other half of the ampicillin resistance gene. This gives pSVgp-DHFR. All these constructs are shown in Figure 19. These

plasmids can be transfected into 3T3 cells or other cells and high levels of gag, pol or env obtained.

An additional method for accomplishing selection is to use a gene selection in one round and its antisense in a subsequent round. For example, gag/pol may be introduced into an HPRT-deficient cell with the HPRT gene and selected for the presence of this gene using that media which requires HPRT for the salvage of purines. In the next round, the antisense to HPRT could be delivered downstream to env and the cell selected in 6 thioguanine for the HPRT-deficient phenotype. Large amounts of antisense HPRT would be required in order to inactivate the HPRT gene transcripts, assuming no reversion occurred.

In addition to the gag/pol expressing constructs which begin at nucleotide 563 of MoMLV, several others can be constructed which contain upstream lead sequences. It has been observed by Prats et al. (RNA Tumor Viruses Meeting, Cold Spring Harbor, N.Y., 1988) that a glycosylated form of the gag protein initiates at nucleotide 357 and a translation enhancer maps in the region between nucleotides 200-270. Therefore, gag/pol expressing constructs may be made beginning at the Sal I site (nucleotide 212) or Eag I site (nucleotide 346) to include these upstream elements and enhance vector production.

Envelope Substitutions

The ability to express gag/pol and env function separately allows for manipulation of these functions independently. A cell line that expresses ample amounts of gag/pol can be used, for example, to address questions of titre with regard to env. One factor resulting in low titres is the density of appropriate receptor molecules on the target cell or tissue. A second factor is the affinity of the receptor for the viral envelope protein. Given that env expression is from a separate unit, a variety of envelope genes (requiring different receptor

proteins), such as xenotropic, polytropic, or amphotrophic envs from a variety of sources, can be tested for highest titres on a specific target tissue. Furthermore, envelopes from nonmurine retrovirus sources can be used for pseudotyping a vector. The exact rules for pseudotyping (i.e., which envelope proteins will interact with the nascent vector particle at the cytoplasmic side of the cell membrane to give a viable viral particle (Tato, Virology 88:71, 1978) and which will not (Vana, Nature 336:36, 1988), are not well characterized. However, since a piece of cell membrane buds off to form the viral envelope, molecules normally in the membrane are carried along on the viral envelope. Thus, a number of different potential ligands can be put on the surface of viral vectors by manipulating the cell line making gag and pol in which the vectors are produced or choosing various types of cell lines with particular surface markers. One type of surface marker that can be expressed in helper cells and that can give a useful vector-cell interaction is the receptor for another potentially pathogenic virus. The pathogenic virus displays on the infected cell surface its virally specific protein (e.g., env) that normally interacts with the cell surface marker or receptor to give viral infection. This reverses the specificity of the infection of the vector with respect to the potentially pathogenic virus by using the same viral protein-receptor interaction, but with the receptors on the vector and the viral protein on the cell.

It may be desirable to include a gene which encodes for an irrelevant envelope protein which does not lead to infection of target cells by the vector so produced, but does facilitate the formation of infectious viral particles. For example, one could use human Sup T1 cells as a helper line. This human T-cell line expresses CD4 molecules at high levels on its surface. Conversion of this into a helper line can be achieved by expressing gag/pol with appropriate expression vectors and also, if

necessary, the Moloney ecotropic env gene product as an irrelevant (for human cells) envelope protein (the Moloney ecotropic env only leads to infection of mouse cells). Vectors produced from such a helper line would have CD4 molecules on their surfaces and are capable of infecting only cells which express HIV env, such as HIV-infected cells.

In addition, hybrid envelopes (as described below) can be used in this system as well, to tailor the tropism (and effectively increase titres) of a retroviral vector. A cell line that expresses ample amounts of a given envelope gene can be employed to address questions of titre with regard to gag and pol.

Cell Lines

The most common packaging cell lines used for MoMLV vector systems (psi2, PA12, PA317) are derived from murine cell lines. There are several reasons why a murine cell line is not the most suitable for production of human therapeutic vectors:

1. They are known to contain endogenous retroviruses.

2. They contain nonretroviral or defective retroviral sequences that are known to package efficiently.

3. There may be deleterious effects caused by the presence of murine cell membrane components.

Several non-murine cell lines are potential packaging lines. These include Vero cells which are used in Europe to prepare polio vaccine, WI38 which are used in the U.S. in vaccine production, CHO cells which are used in the U.S. for TPA preparation and D17 or other dog cells that may have no endogenous viruses.

Although the factors that lead to efficient infection of specific cell types by retroviral vectors are not completely understood, it is clear that because of their relatively high mutation rate, retroviruses may be

adapted for markedly improved growth in cell types in which initial growth is poor, simply by continual reinfection and growth of the virus in that cell type (the adapter cell). This can also be achieved using viral vectors that encode some viral functions (e.g., env), and which are passed continuously in cells of a particular type which have been engineered to have the functions necessary to complement those of the vector to give out infectious vector particles (e.g., gag/pol). For example, one can adapt the murine amphotropic virus 4070A to human T-cells or monocytes by continuous growth and reinfection of either primary cell cultures or permanent cell lines such as Sup T1 (T-cells) or U937 (monocytes). Once maximal growth has been achieved, as measured by reverse transcriptase levels or other assays of virus production, the virus is cloned out by any of a number of standard methods, the clone is checked for activity (i.e., the ability to give the same maximal growth characteristic on transfection into the adapter cell type) and this genome used to make defective helper genomes and/or vectors which in turn, in an appropriately manufactured helper or producer line, will lead to production of viral vector particles which infect and express in the adapter cell type with high efficiency (10^8 - 10^9 infectious units/ml).

VII. Alternative Viral Vector Packaging Techniques

Two additional alternative systems can be used to produce recombinant retroviruses carrying the vector construct. Each of these systems takes advantage of the fact that the insect virus, baculovirus, and the mammalian viruses, vaccinia and adenovirus, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. For example, see Smith et al. (Mol. Cell. Biol. 3:12, 1983); Piccini et al. (Meth. Enzymology, 153:545, 1987); and Mansour et al. (Proc. Natl. Acad. Sci. USA 82:1359, 1985).

These viral vectors can be used to produce proteins in tissue culture cells by insertion of appropriate genes into the viral vector and, hence, could be adapted to make retroviral vector particles.

Adenovirus vectors are derived from nuclear replicating viruses and can be defective. Genes can be inserted into vectors and used to express proteins in mammalian cells either by in vitro construction (Ballay et al., EMBO J. 4:3861, 1985) or by recombination in cells (Thummel et al., J. Mol. Appl. Genetics 1:435, 1982).

One preferred method is to construct plasmids using the adenovirus Major Late Promoter (MLP) driving: (1) gag/pol, (2) env, (3) a modified viral vector construct. A modified viral vector construct is possible because the U3 region of the 5' LTR, which contains the viral vector promoter, can be replaced by other promotor sequences (see, for example, Hartman, Nucl. Acids Res. 16:9345, 1988). This portion will be replaced after one round of reverse transcriptase by the U3 from the 3' LTR.

These plasmids can then be used to make adenovirus genomes in vitro (Ballay et al., op. cit.), and these transfected in 293 cells (a human cell line making adenovirus E1A protein), for which the adenoviral vectors are defective, to yield pure stocks of gag/pol, env and retroviral vector carried separately in defective adenovirus vectors. Since the titres of such vectors are typically 10^{7}-10^{11}/ml, these stocks can be used to infect tissue culture cells simultaneously at high multiplicity. The cells will then be programmed to produce retroviral proteins and retroviral vector genomes at high levels. Since the adenovirus vectors are defective, no large amounts of direct cell lysis will occur and retroviral vectors can be harvested from the cell supernatants.

Other viral vectors such as those derived from unrelated retroviral vectors (e.g., RSV, MMTV or HTV) can be used in the same manner to generate vectors from primary cells. In one embodiment, these adenoviral

vectors are used in conjunction with primary cells, giving rise to retroviral vector preparations from primary cells.

In an alternative system (which is more truly extracellular), the following components are used:

1. gag/pol and env proteins made in the baculovirus system in a similar manner as described in Smith et al. (supra) (or in other protein production systems, such as yeast or E. coli);

2. viral vector RNA made in the known T7 or SP6 or other in vitro RNA-generating system (see, for example, Flamant and Sorge, J. Virol. 62:1827, 1988);

3. tRNA made as in (2) or purified from yeast or mammalian tissue culture cells;

4. liposomes (with embedded env protein); and

5. cell extract or purified necessary components (when identified) (typically from mouse cells) to provide env processing, and any or other necessary cell-derived functions.

Within this procedure (1), (2) and (3) are mixed, and then env protein, cell extract and pre-liposome mix (lipid in a suitable solvent) added. It may, however, be necessary to earlier embed the env protein in the liposomes prior to adding the resulting liposome-embedded env to the mixture of (1), (2), and (3). The mix is treated (e.g., by sonication, temperature manipulation, or rotary dialysis) to allow encapsidation of the nascent viral particles with lipid plus embedded env protein in a manner similar to that for liposome encapsidation of pharmaceuticals, as described in Gould-Fogerite et al., Anal. Biochem. 148:15, 1985). This procedure allows the production of high titres of replication incompetant recombinant retroviruses without contamination with pathogenic retroviruses or replication-competant retroviruses.

VIII. Cell Line-Specific Retroviruses - "Hybrid Envelope"

The host cell range specificity of a retrovirus is determined in part by the env gene products. For example, Coffin, J. (RNA Tumor Viruses 2:25-27, Cold Spring Harbor, 1985) notes that the extracellular component of the proteins from murine leukemia virus (MLV) and Rous Sarcoma virus (RSV) are responsible for specific receptor binding. The cytoplasmic domain of envelope proteins, on the other hand, are understood to play a role in virion formation. While pseudotyping (i.e., the encapsidation of viral RNA from one species by viral protains of another species) does occur at a low frequency, the envelope protein has some specificity for virion formation of a given retrovirus. The present invention recognizes that by creating a hybrid env gene product (i.e., specifically, an env protein having cytoplasmic regions and exogenous binding regions which are not in the same protein molecule in nature) the host range specificity may be changed independently from the cytoplasmic function. Thus, recombinant retroviruses can be produced which will specifically bind to preselected target cells.

In order to make a hybrid protein in which the receptor binding component and the cytoplasmic component are from different retroviruses, a preferred location for recombination is within the membrane-spanning region of the cytoplasmic component. Example 9 describes the construction of a hybrid env gene which expresses a protein with the CD4 binding portion of the HTV envelope protein coupled to the cytoplasmic domain of the MLV envelope protein.

EXAMPLE 9

Hybrid HTV-MLV Envelopes

A hybrid envelope gene is prepared using in vitro mutagenesis (Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492, 1985) to introduce a new restriction site at

an appropriate point of junction. Alternatively, if the two envelope sequences are on the same plasmid, they can be joined directly at any desired point using in vitro mutagenesis. The end result in either case is a hybrid gene containing the 5' end of the HIV gp 160 and the 3' end of MLV p15E. The hybrid protein expressed by the resulting recombinant gene is illustrated in Figure 20 and contains the HIV gp120 (CD4 receptor binding protein), the extracellular portion of HIV gp 41 (the gp 120 binding and fusigenic regions), and the cytoplasmic portion of MLV p15E, with the joint occurring at any of several points within the host membrane. A hybrid with a fusion joint at the cytoplasmic surface (joint C in Figure 20) causes syncytia when expressed in Sup T1 cells. The number of apparent syncytia are approximately one-fifth that of the nonhybrid HIV envelope gene in the same expression vector. Syncytia with the hybrid occurs only when the rev protein is co-expressed in trans. A hybrid with a fusion joint at the extracellular surface (joint A in Figure 20) gives no syncytia while hybrid B (in the middle of the transmembrane regions) gives approximately five-fold less syncytium on Sup T1 cells than hybrid C.

While Example 9 illustrates one hybrid protein produced from two different retroviruses, the possibilities are not limited to retroviruses or other viruses. For example, the beta-receptor portion of human interleukin-2 may be combined with the envelope protein of MLV. In this case, a recombination would preferably be located in the gp 70 portion of the MLV env gene, leaving an intact p15E protein. Furthermore, the foregoing technique may be used to create a recombinant retrovirus with an envelope protein which recognizes antibody Fc segments. Monoclonal antibodies which recognize only preselected target cells only could then be bound to such a recombinant retrovirus exhibiting such envelope proteins so that the retrovirus would bind to and infect only those preselected target cells.

The approach may also be used to achieve tumor-specific targeting and killing by taking advantage of three levels of retroviral vector specificity; namely, cell entry, gene expression, and choice of protein expressed. Retroviral vectors enter cells and exert their effects at intracellular sites. In this respect their action is quite unique. Using this property, and the three levels of natural retroviral specificity (above), retroviral vectors may be engineered to target and kill tumor cells.

The overall goal of targeting of retrovirus to tumor cells may be accomplished by two major experimental routes; namely, a) selection in tissue culture (or in animals) for retroviruses that grow preferentially in tumor cells; or b) construction of retroviral vectors with tissue (tumor) -specific promoters with improvements being made by in vitro passage, and negative and positive drug-sensitivity selection.

At least four selective protocols may be utilized to select for retrovirus which grow preferentially in tumor cells; namely, 1) "Env Selection by Passage In Vitro," wherein selection of retrovirus with improved replicative growth ability is accomplished by repeated passage in tumor cells; 2) "Selection with a Drug Resistance Gene," wherein genetic selection for tumor "specific" retroviruses is based on viral constructs containing a linked drug resistance gene; 3) "Hybrid-Env," wherein selection (by protocol #1 or #2, above) of retrovirus with tumor-"specificity" is initiated from a construct containing a hybrid envelope gene which is a fusion of a tumor receptor gene (i.e., an anti-tumor antibody H-chain V-region gene fused with env; or, a growth receptor fused with env); in this case selection begins at a favorable starting point, e.g., an env which has some specificity for tumor cells; or 4) "Selection by Passage In Vitro and Counter Selection by Co-cultivation with Normal Cells," wherein growth in tumor cells is

selected-for by repeated passage in mixtures of drug-resistant tumor cells and drug-sensitive normal cells.

With respect to retroviral vector constructs carrying tissue (tumor) -specific promoters, biochemical markers with different levels of tissue-specificity are well known, and genetic control through tissue-specific promoters is understood in some systems. There are a number of genes whose transcriptional promoter elements are relatively active in rapidly growing cells (i.e., transferrin receptor, thymidine kinase, etc.) and others whose promoter/enhancer elements are tissue specific (i.e., HBV enhancer for liver, PSA promoter for prostate). Retroviral vectors and tissue-specific promoters (present either as an internal promoter or within the LTR) which can drive the expression of selectable markers and cell cycle genes (i.e., drug sensitivity, Eco gpt; or HSVTK in TK-cells). Expression of these genes can be selected for in media containing mycophenolic acid or HAT, respectively. In this manner, tumor cells containing integrated provirus which actively expresses the drug resistance gene will survive. Selection in this system may involve selection for both tissue-specific promoters and viral LTRs. Alternatively, specific expression in tumor cells, and not in normal cells, can be counter-selected by periodically passaging virus onto normal cells, and selecting against virus that express Eco gpt or HSVtk (drug sensitivity) in those cells (by thioxanthine or acyclovir). Infected cells containing integrated provirus which express Eco gpt or tk phenotype will die and thus virus in that cell type will be selected against.

IX. Site-Specific Integration

Targeting a retroviral vector to a predetermined locus on a chromosome increases the benefits of gene-delivery systems. A measure of safety is gained by direct integration to a "safe" spot on a chromosome, i.e., one that is proven to have no deleterious effects from the

insertion of a vector. Another potential benefit is the ability to direct a gene to an "open" region of a chromosome, where its expression would be maximized. Two techniques for integrating retroviruses at specific sites are described below.

(i) Homologous Recombination

One technique for integrating an exogenous gene of a vector construct of a recombinant retrovirus into a specific site in a target cell's DNA employs homologous recombination. Plasmids containing sequences of DNA of greater than about 300 bp that are homologous to genomic sequences have been shown to interact (either by replacement or insertion) with those genomic sequences at a rate that is greater than 10^3-fold over a specific interaction in the absence of such homology (see Thomas and Capecchi, Cell 51:503-12, 1987; and Doetscheman et al., Nature 330:576-78, 1987). It has been shown that an insertion event, or alternatively, a replacement event, may be driven by the specific design of the vector.

In order to employ homologous recombination in site-specific retroviral integration, a vector construct should be modified such that (a) homologous sequences (to the target cell's genome) are incorporated into the construct at an appropriate location; and (b) the normal mechanism of integration does not interfere with the targeting occurring due to homologous sequences. A preferred approach in this regard is to add homologous sequences (greater than about 300 bp) in the 3' LTR, downstream from the U3 inverted repeat. In this approach, the construct is initially made with a region of homology inserted in the 3' LTR at the Nhe I site in U3. Reverse transcription in the host cell will result in a duplication of the region of homology in the 5' LTR within 31 bp of the end of the inverted repeat (IR). Integration into the host genome will occur in the presence or absence of the normal integration mechanism. The gene in the

vector may be expressed, whether from the LTR or from an internal promoter. This approach has the effect of placing a region of homology near a potential free end of the double-stranded retrovirus vector genome. Free ends are known to increase the frequency of homologous recombination by a factor of approximately ten. In this approach, it may be necessary to defeat the normal mechanism of integration, or to at least modify it to slow down the process, allowing time for homologous DNAs to line up. Whether this latter modification is required in a particular case can be readily ascertained by one skilled in the art.

(ii) Integrase Modification

Another technique for integrating a vector construct into specific, preselected sites of a target cell's genome involves integrase modification.

The retrovirus pol gene product is generally processed into four parts: (i) a protease which processes the viral gag and pol products; (ii) the reverse transcriptase; and (iii) RNase H, which degrades RNA of an RNA/DNA duplex; and (iv) the endonuclease or "integrase."

The general integrase structure has been analyzed by Johnson et al. (Proc. Natl. Acad. Sci. USA 83:7648-7652, 1986). It has been proposed that this protein has a zinc binding finger with which it interacts with the host DNA before integrating the retroviral sequences.

In other proteins, such "fingers" allow the protein to bind to DNA at particular sequences. One illustrative example is the steroid receptors. In this case, one can make the estrogen receptor, responding to estrogens, have the effect of a glucocorticoid receptor, responding to glucocorticoids, simply by substituting the glucocorticoid receptor "finger" (i.e., DNA binding segment) in place of the estrogen receptor finger segment in the estrogen receptor gene. In this example, the

position in the genome to which the proteins are targeted has been changed. Such directing sequences can also be substituted into the integrase gene in place of the present zinc finger. For instance, the segment coding for the DNA binding region of the human estrogen receptor gene may be substituted in place of the DNA binding region of the integrase in a packaging genome. Initially, specific integration would be tested by means of an in vitro integration system (Brown et al., Cell 29:347-356, 1987). To confirm that the specificity would be seen in vivo, this packaging genome is used to make infectious vector particles, and infection of and integration into estrogen-sensitive and estrogen-nonsensitive cells compared in culture.

Through use of this technique, incoming viral vectors may be directed to integrate into preselected sites on the target cell's genome, dictated by the genome-binding properties of site-specific DNA-binding protein segments spliced into the integrase genome. It will be understood by those skilled in the art that the integration site must, in fact, be receptive to the fingers of the modified integrase. For example, most cells are sensitive to glucocorticoids and hence their chromatin has sites for glucocorticoid receptors. Thus, for most cells, a modified integrase having a glucocorticoid receptor finger would be suitable to integrate the proviral vector construct at those glucocorticoid receptor-binding sites.

X. Production of Recombinant Retroviral Vectors in Transgenic Animals

Two problems previously described with helper line generation of retroviral vectors are: (a) difficulty in generating large quantities of vectors; and (b) the current need to use permanent instead of primary cells to make vectors. These problems can be overcome with producer or packaging lines that are generated in

transgenic animals. These animals would carry the packaging genomes and retroviral vector genomes. Current technology does not allow the generation of packaging cell lines and desired vector-producing lines in primary cells due to their limited life span. The current technology is such that extensive characterization is necessary, which eliminates the use of primary cells because of senescence. However, individual lines of transgenic animals can be generated by the methods provided herein which produce the packaging functions, such as gag, pol or env. These lines of animals are then characterized for expression in either the whole animal or targeted tissue through the selective use of housekeeping or tissue-specific promoters to transcribe the packaging functions. The vector to be delivered is also inserted into a line of transgenic animals with a tissue-specific or housekeeping promoter. As discussed above, the vector can be driven off such a promoter substituting for the U3 region of the 5' LTR (Figure 21). This transgene could be inducible or ubiquitous in its expression. This vector, however, is not packaged. These lines of animals are then mated to the gag/pol/env animal and subsequent progeny produce packaged vector. The progeny, which are essentially identical, are characterized and offer an unlimited source of primary producing cells. Alternatively, primary cells making gag/pol and env and derived from transgenic animals can be infected or transfected in bulk with retrovirus vectors to make a primary cell producer line. Many different transgenic animals or insects could produce these vectors, such as mice, rats, chickens, swine, rabbits, cows, sheep, fish and flies. The vector and packaging genomes would be tailored for species infection specificity and tissue-specific expression through the use of tissue-specific promoters and different envelope proteins. An example of such a procedure is illustrated in Figure 22.

Although the following examples of transgenic production of primary packaging lines are described only for mice, these procedures can be extended to other species by those skilled in the art. These transgenic animals may be produced by microinjection or gene transfer techniques. Given the homology to MLV sequences in mice genome, the final preferred animals would not be mice.

EXAMPLE 10

Production of Gag/Pol Proteins Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

An example of a well-characterized housekeeping promoter is the HPRT promoter. HPRT is a purine salvage enzyme which expresses in all tissues. (See Patel et al., Mol. Cell Biol. 6:393-403, 1986 and Melton et al., Proc. Natl. Acad. Sci. 81:2147-2151, 1984). This promoter is inserted in front of various gag/pol fragments (e.g., Bal I/Sca I; Aat II/Sca I; Pst I/Sca I of MoMLV; see RNA Tumor Viruses 2, Cold Spring Harbor Laboratory, 1985) that are cloned in Bluescript plasmids (Strategene, Inc.) using recombinant DNA techniques (see Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982). The resulting plasmids are purified (Maniatis et al., op. cit.) and the relevant genetic information isolated using Geneclean (Bio 101) or electroelution (see Hogan et al. (eds.), Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, 1986).

These fully characterized DNAs are microinjected in the pronucleus of fertilized mouse ova at a concentration of 2 ug/ml. Live-born mice are screened by tail blot analyses (see Hogan et al., op. cit.). Transgenic-positive animals are characterized for expression levels of gag-pol protains by immunoprecipitation of radiolabeled primary cells, such as fibroblast (see Harlow et al. (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor, 1988). Animals then bred to

homozygosity for establishment of animal lines that produce characterized levels of gag-pol.

EXAMPLE 11

Production of env Proteins/Hybrid Envelope Proteins Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

This example utilizes the HPRT promoter for expression of either envelope or hybrid envelope proteins. The envelope proteins can be from any retrovirus that is capable of complementing the relevant gag-pol, in this case that of MLV. Examples are ecotropic MLV, amphotrophic MLV, xenotropic MLV, polytropic MLV, or hybrid envelopes. As above, the envelope gene is cloned behind the HPRT promoter using recombinant DNA techniques (see Maniatis et al., op. cit.). The resulting "minigene" is isolated (see Hogan et al., op. cit.), and expression of envelope protein is determined (Harlow et al., op. cit.). The transgenic envelope animals are bred to homozygosity to establish a well-characterized envelope animal.

EXAMPLE 12

Production of gag-pol-env Animals Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

This uses the well-characterized gag-pol animals, as well as the animals for the establishment of a permanent gag-pol/envelope animal line. This involves breeding to homozygosity and the establishment of a well-characterized line. These lines are then used to establish primary mouse embryo lines that can be used for packaging vectors in tissue culture. Furthermore, animals containing the retroviral vector are bred into this line.

EXAMPLE 12

Production of Tissue-Specific Expression of Gag-pol-env or Hybrid Envelope in Transgenic Animals

This example illustrates high level expression of the gag/pol, envelope, or hybrid envelope in specific tissues, such as T-cells. This involves the use of CD2 sequences (see Lang et al., EMBO J. 7:1675-1682, 1988) that give position and copy number independence. The 1.8 kb Bam HI/Hind III fragment from the CD2 gene is inserted in front of gag-pol, envelope, or hybrid envelope fragments using recombinant DNA techniques. These genes are inserted into fertilized mouse ova by microinjection. Transgenic animals are characterized as before. Expression in T-cells is established, and animals are bred to homozygosity to establish well-characterized lines of transgenic animals. Gag-pol animals are mated to envelope animals to establish gag-pol-env animals expressing only in T-cells. The T-cells of these animals are then a source for T-cells capable of packaging retroviral vectors. Again, vector animals can be bred into these gag-pol-env animals to establish T-cells expressing the vector.

This technique allows the use of other tissue-specific promoters, such as milk-specific (whey), pancreatic (insulin or elastase), or neuronal (myelin basic protein) promoters. Through the use of promoters, such as milk-specific promoters, recombinant retroviruses may be isolated directly from the biological fluid of the progeny.

EXAMPLE 13

Production of Either Housekeeping or Tissue-Specific Retroviral Vectors in Transgenic Animals

The insertion of retroviruses or retroviral vectors into the germ line of transgenic animals results in little or no expression. This effect, described by

Jaenisch (see Jahner et al., Nature 298:623-628, 1982), is attributed to methylation of 5' retroviral LTR sequences. This technique would overcome the methylation effect by substituting either a housekeeping or tissue-specific promoter to express the retroviral vector/retrovirus. The U3 region of the 5' LTR, which contains the enhancer elements, is replaced with regulatory sequences from housekeeping or tissue-specific promoters (see Figure 20). The 3' LTR is fully retained, as it contains sequences necessary for polyadenylation of the viral RNA and integration. As the result of unique properties of retroviral replication, the U3 region of the 5' LTR of the integrated provirus is generated by the U3 region of the 3' LTR of the infecting virus. Hence, the 3' is necessary, while the 5' U3 is dispensable. Substitution of the 5' LTR U3 sequences with promoters and insertion into the germ line of transgenic animals results in lines of animals capable of producing retroviral vector transcripts. These animals would then be mated to gag-pol-env animals to generate retroviral-producing animals (see Figure 22).

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A recombinant retrovirus carrying a vector construct which directs the expression in target cells infected with the retrovirus of at least one protein or modified form thereof being capable of stimulating an immune response within an animal, the vector construct further comprising a segment encoding a viral capsid protein and a regulatory response element, and a segment encoding at least one regulatory element.

2. The recombinant retrovirus of claim 1 wherein the viral capsid protein is the HIV gag protein, the regulatory element is the HIV rev protein, and the regulatory response element is the HIV rev responsive regulatory element.

3. The recombinant retrovirus of claim 1 wherein the vector construct directs the expression of at least one HIV protein selected from the group consisting of HIV gag, HIV pol, HIV rev, HIV vpu, HIV vif, HIV and HIV prot.

4. The recombinant retrovirus of claim 1 wherein the segment encoding the viral capsid protein is HIV gag, the segment being operably linked to a second gene, wherein the HIV gag and second gene are capable of directing the expression of the protein or modified form thereof.

5. A recombinant retrovirus carrying a vector construct having a tissue tumor-specific transcriptional promoter/enhancer element, the construct directing the expression of a palliative in cells having a tissue type compatible with the promoter, said palliative being capable of inhibiting a function of a pathogenic agent necessary for pathogenicity.

6. A recombinant retrovirus carrying a vector construct which directs the expression of a peptide having multiple epitopes, one or more of said epitopes being derived from different proteins.

7. A recombinant retrovirus carrying a vector construct which directs the expression of peptidic antigenic fragment or modified form thereof in target cells infected with the retrovirus, said antigenic fragment or modified form thereof being capable of stimulating an immune response within an animal.

8. A recombinant retrovirus carrying a vector construct which directs the expression of at least one antigen or modified form thereof and an MHC protein or modified form thereof in target cells infected with the retrovirus, said antigen or modified form thereof and the MHC protein or modified form thereof being capable of stimulating an immune response within an animal.

9. A recombinant retrovirus carrying a vector construct which directs the expression of a first antigen or modified form thereof in target cells infected with the retrovirus, said first antigen being capable of stimulating an immune response within an animal against a second antigen or modified form thereof, wherein the first and the second antigens are related but not identical.

10. The recombinant retrovirus of claim 9 wherein the first and second antigens or modified forms thereof share at least 40% homology, but not more than 99% homology, in a sequence of 8 to 100 amino acids.

11. A recombinant retrovirus carrying a vector construct which directs the expression of at least one antigen or modified form thereof in target cells infected with the retrovirus, said antigen or modified form thereof being capable of stimulating an immune response *in vitro*.

12. A DNA vector construct which directs the expression of at least one antigen or modified form thereof in target cells transfected with the DNA, said antigen or modified form thereof being capable of stimulating an immune response *in vitro*.

13. The recombinant retrovirus of claims 11 or 12 wherein the *in vitro* immune response is a cell-mediated immune response.

14. The recombinant retrovirus of claims 11 or 12 wherein the *in vitro* immune response is a CTL response.

15. A recombinant retrovirus carrying a vector construct which directs the transient expression of at least one antigen or modified form thereof in target cells infected with the retrovirus, said antigen or modified form thereof being capable of stimulating an immune response within an animal.

16. A DNA vector construct which directs the transient expression of at least one antigen or modified form thereof in target cells transfected with the DNA, said antigen or modified form thereof being capable of stimulating an immune response within an animal.

17. A recombinant retrovirus carrying a vector construct which directs the expression of a palliative in cells infected with the retrovirus, the palliative being capable of inhibiting the activity of a protein produced by a pathogenic agent.

18. A recombinant retrovirus carrying a vector construct which directs the expression of an RNA molecule which functions as a ribozyme specific for a pathogenic RNA molecule.

19. The recombinant retrovirus of any of claims 1-18 wherein said retrovirus is replication defective.

20. A method of producing a recombinant retrovirus, comprising:

packaging a vector construct in a capsid and envelope such that a replication defective recombinant retrovirus according to claim 19 is produced.

21. *Ex vivo* cells infected with a recombinant retrovirus or transfected with a DNA vector construct according to any of claims 1-19.

22. Eucaryotic cells infected with a recombinant retrovirus according to any of claims 1-11 and 13-18, said cells being capable of generating infectious particles containing any one of said vector constructs.

23. A pharmaceutical composition comprising a retrovirus according to any one of claims 1-11 and 13-18, in combination with a physiologically acceptable carrier or diluent.

24. The pharmaceutical composition of claim 21, for use as an active therapeutic substance.

25. A method of identifying an antigen or antigenic epitopes in a protein, reactive with T lymphocytes, comprising:

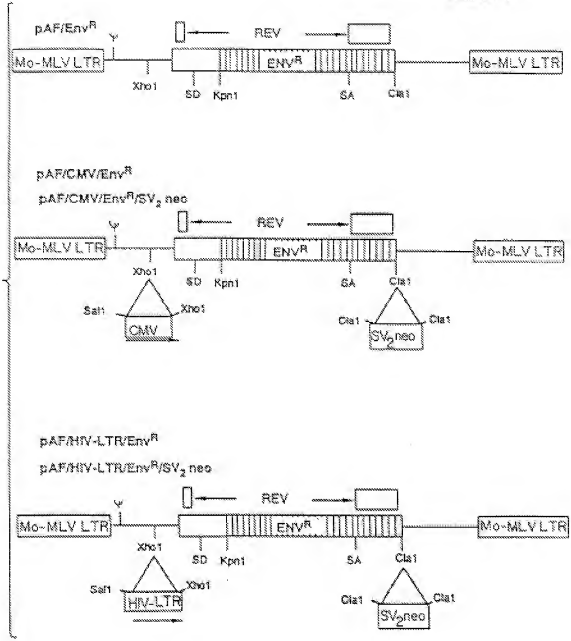
(a) preparing a multiplicity of different recombinant retroviruses each carrying a recombinant vector construct directing the expression of a different antigen, or peptidic fragment of the antigen;

(b) infecting a multiplicity of target cells with the different recombinant retroviruses; and

(c) testing for the ability of CTL or antibodies to kill target cells expressing the different antigens or peptidic fragments of the antigen, and therefrom determining which antigen or peptidic fragments of the antigen are reactive with the T lymphocytes.

RETROVIRAL CONSTRUCTS OF ENV^R

FIG. 1



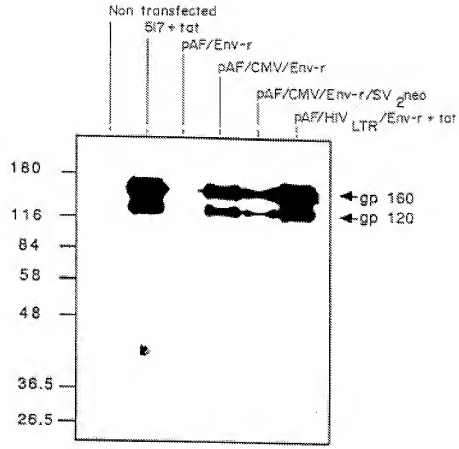

Non transfected
5i7 + tat
pAF/Env-r
pAF/CMV/Env-r
pAF/CMV/Env-r/SV 2neo
pAF/HIV LTR/Env-r + tat
180
116
84
58
48
36.5
26.5
gp 160
gp 120

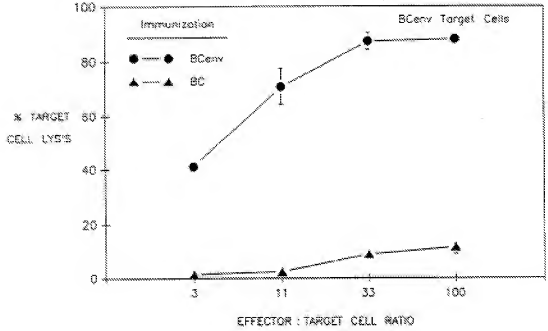
FIG.2

FIG. 3

Induction of Anti-HIV env CTL in Balb/c Mice
Using Retroviral-Infected Stimulator Cells

FIG. 4A

INDUCTION OF CYTOTOXIC EFFECTORS IN MICE IMMUNIZED WITH BCenv STIMULATOR CELLS



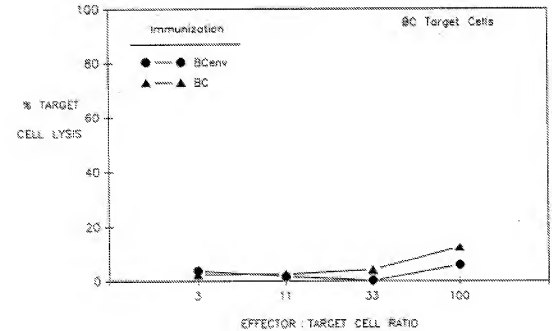


FIG. 4B

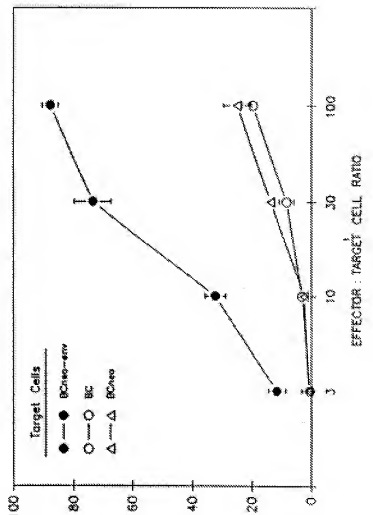

SPECIFICITY OF ANTI-BCenv CTL EFFECTORS
Target Cells
BCneo-env
BC
BCneo
% TARGET CELL LYSIS
100
80
60
40
20
0
3
10
30
100
EFFECTOR : TARGET CELL RATIO

FIG. 4C

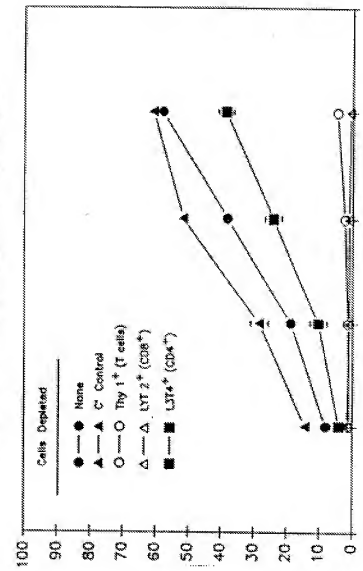

PHENOTYPE OF ANTI-BCenv EFFECTOR CELLS
Cells Depleted
None
C' Control
Thy 1+ (T cells)
LYT 2+ (CD8+)
L3T4+ (CD4+)
% TARGET CELL LYSIS
100
90
80
70
60
50
40
30
20
10
0
1
3
10
30
EFFECTOR : TARGET CELL RATIO

FIG. 4D

MHC RESTRICTION REQUIREMENTS FOR ANTI-BCony CTL RESPONSE : INHIBITION BY POLYCLONAL ANTI-MHC ANTISERA

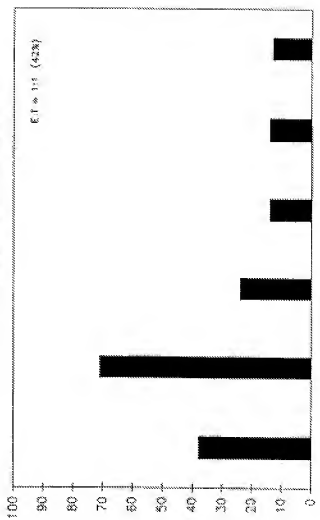
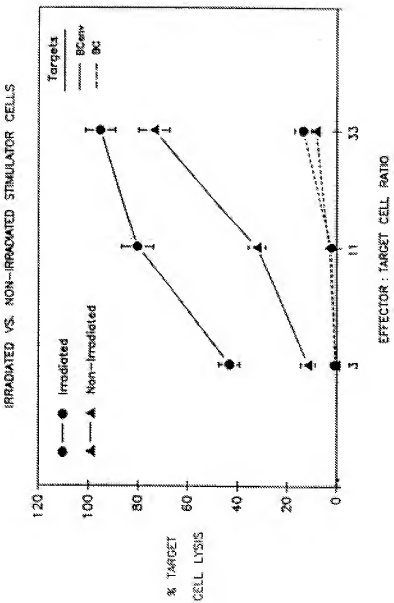


FIG 4E


ANTI-BCenv CTL INDUCTION :
IRRADIATED VS. NON-IRRADIATED STIMULATOR CELLS
Irradiated
Non-Irradiated
Targets
BCenv
BC
% TARGET CELL LYSIS
120
100
80
60
40
20
0
3
11
33
EFFECTOR : TARGET CELL RATIO

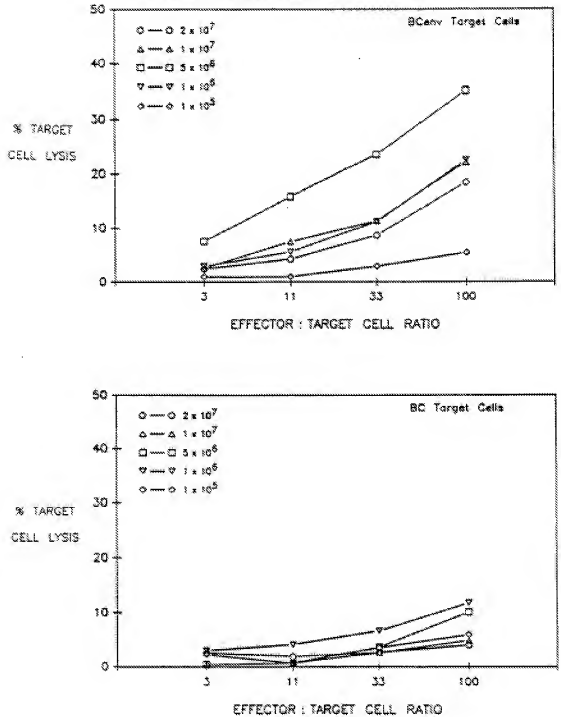
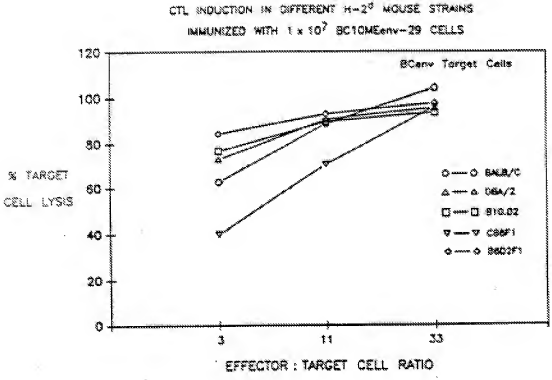

CTL INDUCTION IN BALB/C MICE IMMUNIZED WITH
VARYING CONCENTRATIONS OF BCenv STIMULATOR CELLS
BCenv Target Cells
2 x 10^7
1 x 10^7
5 x 10^6
1 x 10^6
1 x 10^5
% TARGET CELL LYSIS
50
40
30
20
10
0
3
11
33
100
EFFECTOR : TARGET CELL RATIO
BC Target Cells
2 x 10^7
1 x 10^7
5 x 10^6
1 x 10^6
1 x 10^5
% TARGET CELL LYSIS
50
40
30
20
10
0
3
11
33
100
EFFECTOR : TARGET CELL RATIO

FIG. 4F


CTL INDUCTION IN DIFFERENT H-2^d MOUSE STRAINS
IMMUNIZED WITH 1 x 10^7 BC10MEenv-29 CELLS
BCenv Target Cells
% TARGET CELL LYSIS
120
100
80
60
40
20
0
3
11
33
EFFECTOR : TARGET CELL RATIO
BALB/C
DBA/2
B10.D2
C3BF1
B6D2F1

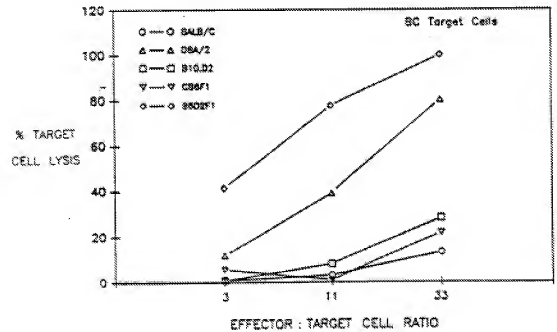

BC Target Cells
BALB/C
DBA/2
B10.D2
C3BF1
B6D2F1
% TARGET CELL LYSIS
120
100
80
60
40
20
0
3
11
33
EFFECTOR : TARGET CELL RATIO

FIG. 4G

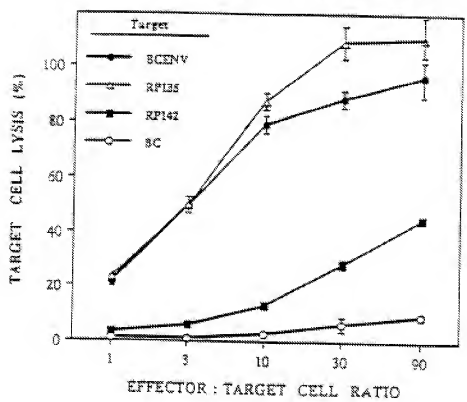

CROSSREACTIVITY OF BCenv-INDUCED CTL
Target
BCENV
RP135
RP142
BC
TARGET CELL LYSIS (%)
0
20
40
60
80
100
1
3
10
30
90
EFFECTOR : TARGET CELL RATIO

FIG. 4H

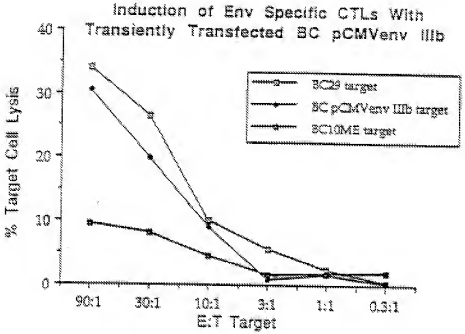

Induction of Env Specific CTLs With
Transiently Transfected BC pCMVenv IIIb
% Target Cell Lysis
40
30
20
10
0
90:1
30:1
10:1
3:1
1:1
0.3:1
E:T Target
BC29 target
BC pCMVenv IIIb target
BC10ME target

FIG. 4I

Transient Infection for Constructing Target Cells
C57BL/6 anti-H2-Dd CTL on MHC-Dd transfected murine BC target cells
3T3
3T3CMV-Dd
3T3RSV-Dd
% Cell Lysis
Effector : Target Cell Ratio
0.3
1
3
11
33
100
0
10
20
30
40
50
60
70

FIG. 4J

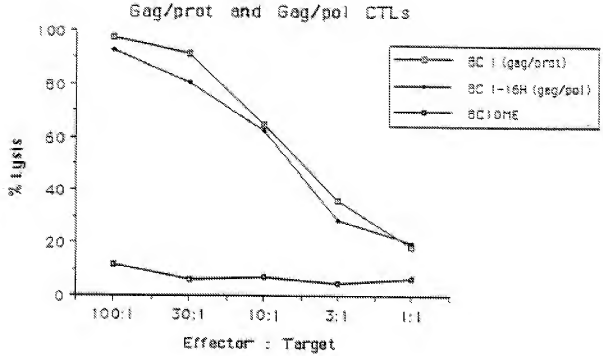

Gag/prot and Gag/pol CTLs
% Lysis
100
80
60
40
20
0
100:1
30:1
10:1
3:1
1:1
Effector : Target
BC 1 (gag/prot)
BC 1-16H (gag/pol)
BC10ME

FIG.4K

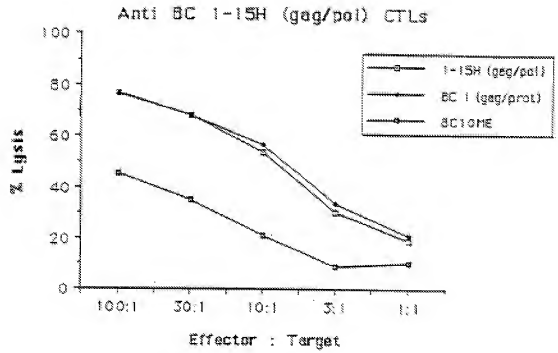

Anti BC 1-15H (gag/pol) CTLs
100
80
60
40
20
0
% Lysis
100:1
30:1
10:1
3:1
1:1
Effector : Target
1-15H (gag/pol)
BC 1 (gag/prot)
BC1GHE

FIG.4L

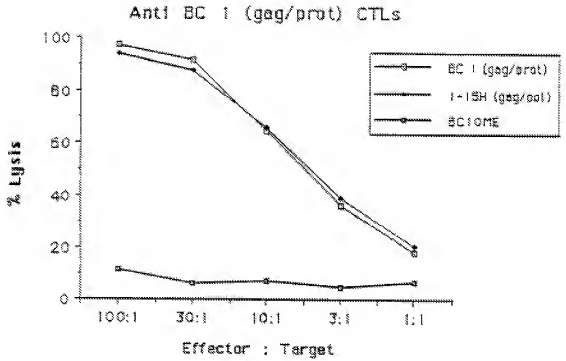

Anti BC 1 (gag/prot) CTLs
100
80
60
40
20
0
% Lysis
100:1
30:1
10:1
3:1
1:1
Effector : Target
BC 1 (gag/prot)
1-1SH (gag/pol)
BC1OME

FIG.4M

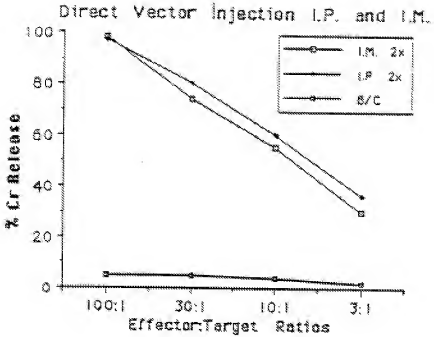

Direct Vector Injection I.P. and I.M.
100
80
60
40
20
0
% Cr Release
I.M. 2x
I.P. 2x
B/C
100:1
30:1
10:1
3:1
Effector:Target Ratios

FIG. 4N

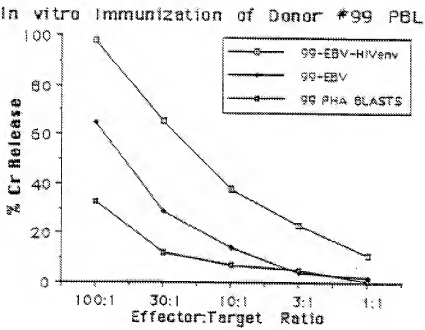

In vitro Immunization of Donor #99 PBL
% Cr Release
100
80
60
40
20
0
99-EBV-HIVenv
99-EBV
99 PHA BLASTS
100:1
30:1
10:1
3:1
1:1
Effector:Target Ratio

FIG.40

INFECTION OF TARGET CELLS WITH MHC AND ANTIGEN.

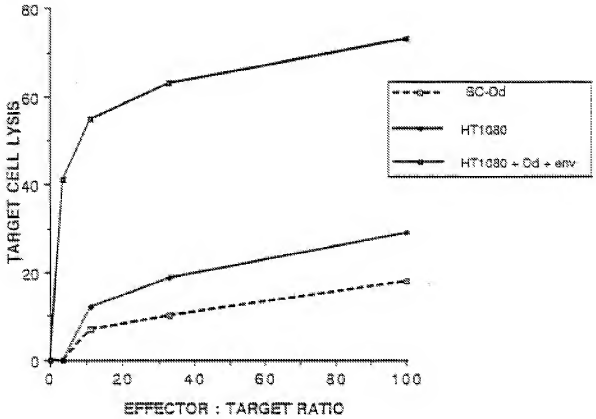
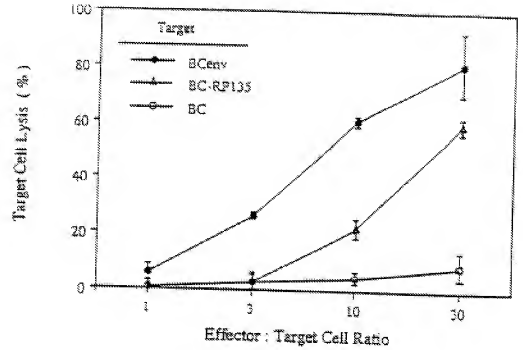


FIG. 4P

A. CTL Induction with BCenv



B. CTL Induction with BCenvΔV3

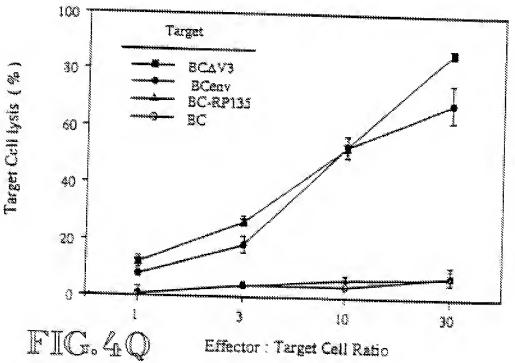
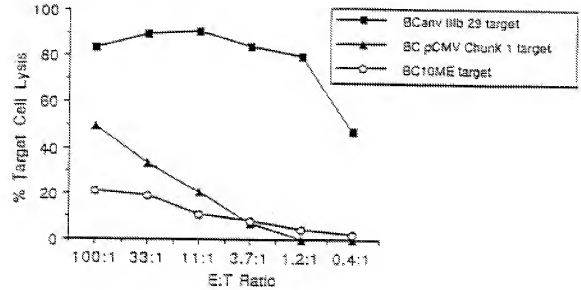


FIG. 4Q

A. Lysis of BCpCMV Chunk 1 Target



B. Induction of Balb/c anti-Chunk 1 CTLs

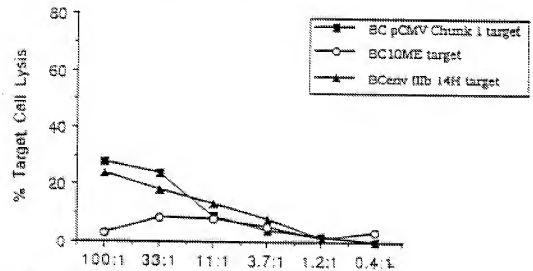


FIG. 4R

RECEPTOR BLOCKER VECTOR

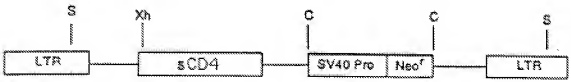


FIG.5

Construction of retroviral vectors pTK - 1 and pTK - 3

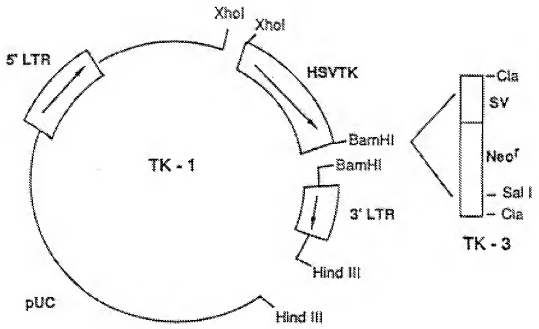


FIG.6

Construction of HIV-conditionally - lethal vector KTVIHAX

FIG. 7

Construction of KTVIH5 and KTVIH Neo

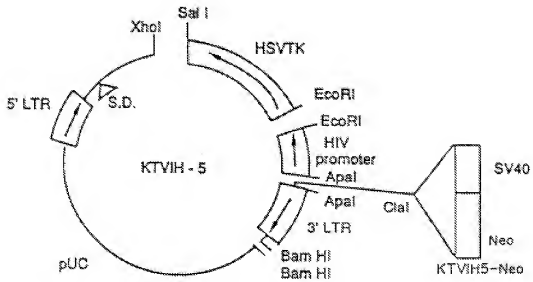


FIG.8

CONSTRUCTION OF MHMTKNEO RETROVIRAL VECTOR

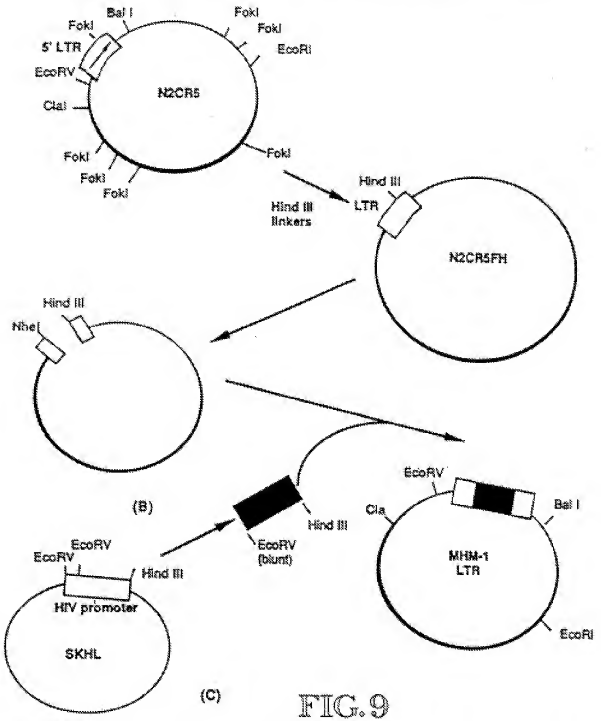
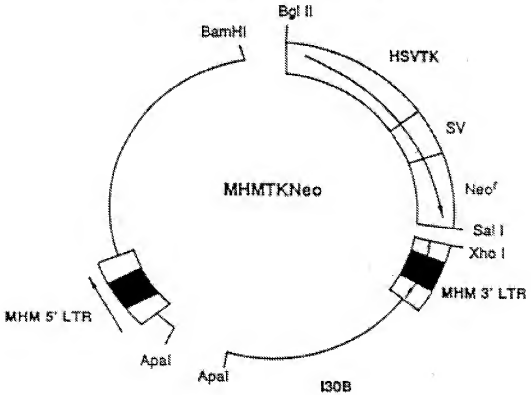


FIG. 9

FIG. 9 CONT.


Bgl II
BamHI
HSVTK
SV
MHMTKNeo
Neo^r
Sal I
Xho I
MHM 3' LTR
MHM 5' LTR
ApaI
ApaI
I30B

Construction of HIV tat- and HIV rev-responsive conditionally lethal vector, RRKTVIH

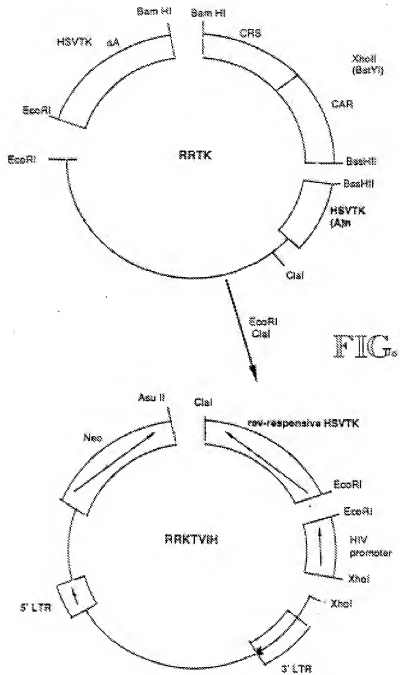
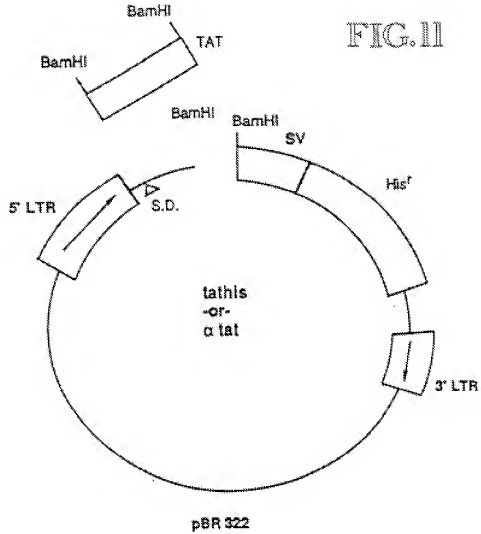


FIG. 10

FIG. 11


BamHI
TAT
BamHI
BamHI
BamHI
SV
His^r
5' LTR
S.D.
tathis
-or-
α tat
3' LTR
pBR 322

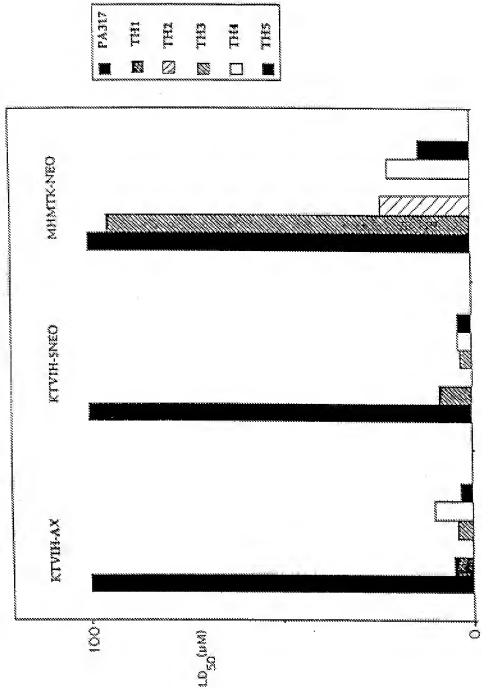

ACV Toxicity in Cells Containing Conditional Lethal Vectors
KTVIH-AX
KTVIH-SNEO
MHMTK-SNEO
PA317
TH1
TH2
TH3
TH4
TH5
100
0
LD_{50} (μM)
Cell Line

FIG. 12

Construction of vector 4TVIHAX

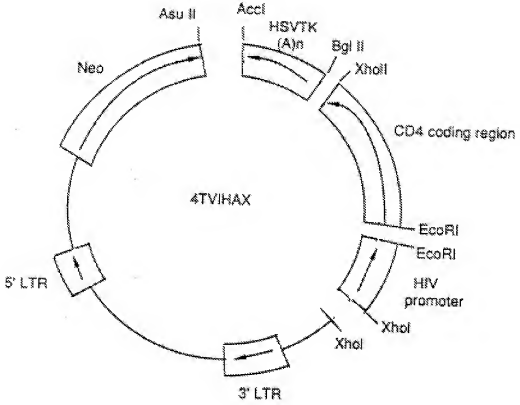


FIG. 13

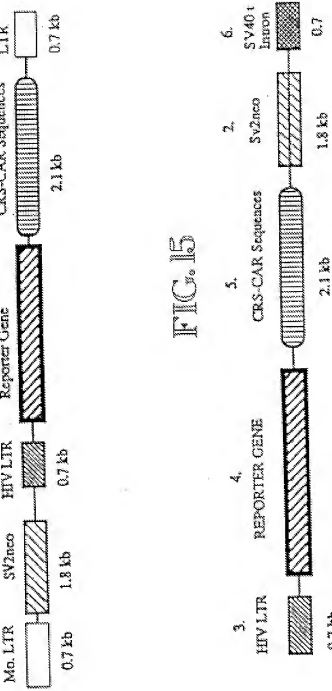

1.
Mo. LTR
0.7 kb
2
SV2neo
1.8 kb
3.
HIV LTR
0.7 kb
4.
Reporter Gene
5.
CRS-CAR Sequences
2.1 kb
1.
Mo. LTR
0.7 kb

FIG. 14

3.
HIV LTR
0.7 kb
4.
REPORTER GENE
5.
CRS-CAR Sequences
2.1 kb
2.
SV2neo
1.8 kb
6.
SV40 t Intron
0.7

FIG. 15

FIG. 16

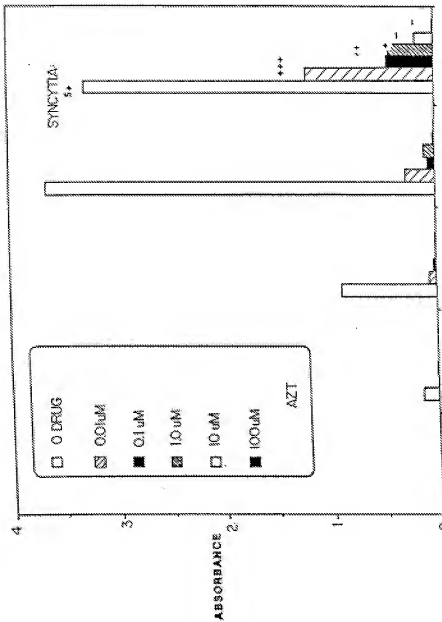

EFFECT OF AZT ON HIV INFECTION
ABSORBANCE
DAY
SYNCYTIA-
AZT
0 DRUG
0.01uM
0.1 uM
1.0 uM
10 uM
100uM

FIG. 17

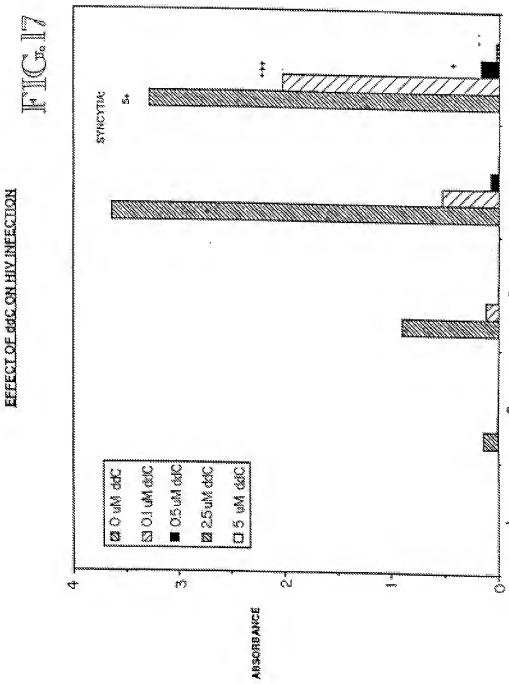
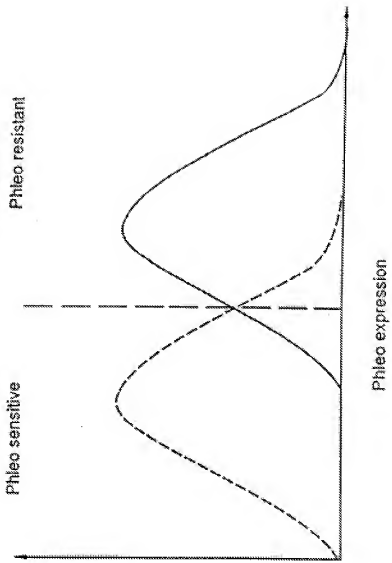

EFFECT OF ddC ON HIV INFECTION
ABSORBANCE
DAY
SYNCYTIA
0 uM ddC
0.1 uM ddC
0.5 uM ddC
2.5 uM ddC
5 uM ddC
++
+
-

FIG. 18


# of transfectants
Phleo sensitive
Phleo resistant
Phleo expression

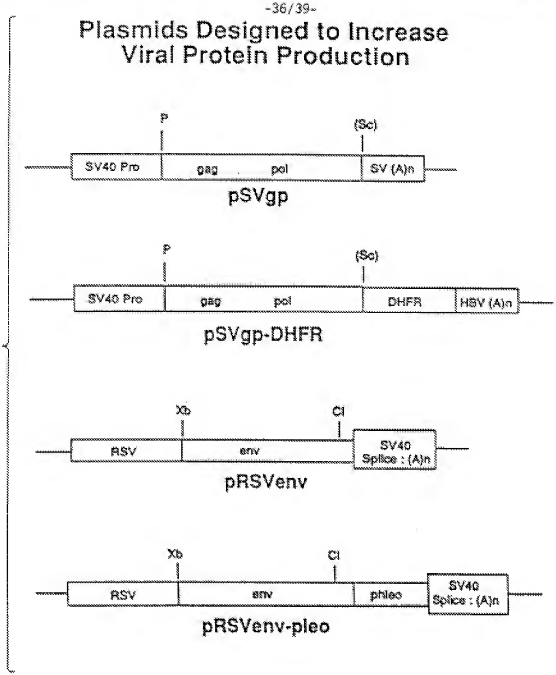
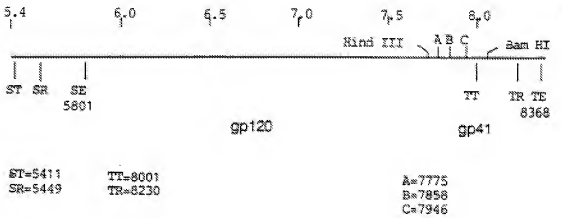

Plasmids Designed to Increase
Viral Protein Production
P
(Sc)
SV40 Pro
gag
pol
SV (A)n
pSVgp
P
(Sc)
SV40 Pro
gag
pol
DHFR
HSV (A)n
pSVgp-DHFR
Xb
Cl
RSV
env
SV40
Splice : (A)n
pRSVenv
Xb
Cl
RSV
env
phleo
SV40
Splice : (A)n
pRSVenv-pleo

FIG. 19

CREATION OF FUSION SITES ON HIV AND MLV ENV GENES - 37/39-

HIV



MoMLV

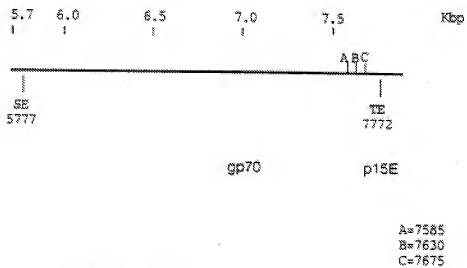


FIG. 20

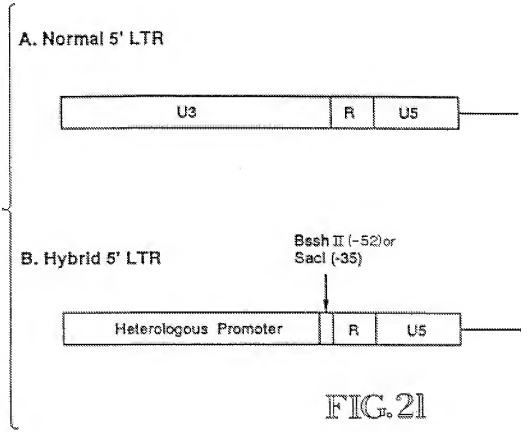
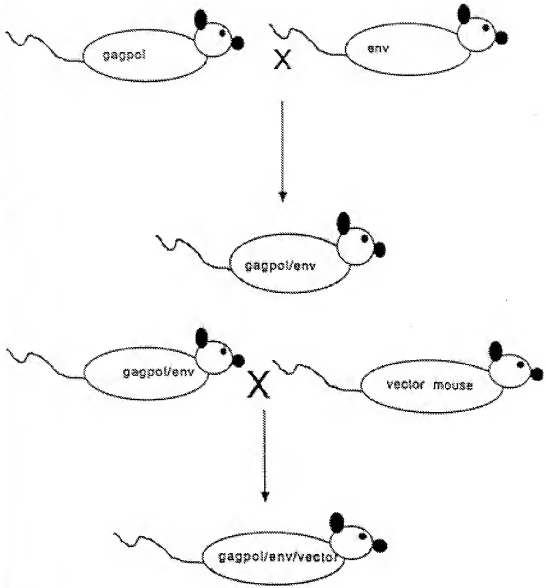

A. Normal 5' LTR
U3
R
U5
B. Hybrid 5' LTR
BsshII (-52) or
SacI (-35)
Heterologous Promoter
R
U5

FIG. 21

FIG. 22


gagpol
X
env
gagpol/env
gagpol/env
X
vector mouse
gagpol/env/vector